

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 15/11, 15/12, A61K 48/00	A1	(11) International Publication Number: WO 92/22651 (43) International Publication Date: 23 December 1992 (23.12.92)
(21) International Application Number: PCT/US92/05008 (22) International Filing Date: 11 June 1992.(11.06.92) (30) Priority data: 715,196 14 June 1991 (14.06.91) US (71) Applicant: ISIS PHARMACEUTICALS, INC. [US/US]; 2280 Faraday Avenue, Carlsbad, California 92008 (US). (72) Inventors: MONIA, Brett, P. ; 6525-G Paseo Frontera, Carlsbad, CA 92009 (US). FREIER, Susan, M. ; 2946 Renault Street, San Diego, California 92122 (US). ECK- ER, David, J. ; 2609 Colibri Lane, Carlsbad, CA 92009 (US).		(74) Agents: CALDWELL, John, W. et al.; Woodcock Wash- burn Kurtz Mackiewicz & Norris, One Liberty Place - 46th Floor, Philadelphia, PA 19103 (US). (81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE (European patent), DK (European pa- tent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European pa- tent), JP, KP, KR, LK, LU (European patent), MC (Eu- ropean patent), MG, ML (OAPI patent), MN, MR (OA- PI patent), MW, NL (European patent), NO, PL, RO, RU, SD, SE (European patent), SN (OAPI patent), TD (OAPI patent), TG (OAPI patent). Published <i>With international search report.</i>
(54) Title: ANTISENSE OLIGONUCLEOTIDE INHIBITION OF THE RAS GENE (57) Abstract Compositions and methods are provided for the modulation of expression of the human ras gene in both the normal and activated forms. Oligonucleotides are provided which are specifically hybridizable with RNA or DNA deriving from the human ras gene, having nucleotide units sufficient in identity and number to effect such specific hybridization. Oligonucleotides or oligonucleotide analogs specifically hybridizable with a translation initiation site or with the codon-12 mutation of activated ras are provided. Such oligonucleotides and oligonucleotide analogs can be used for diagnostics as well as for research purposes. Methods are also disclosed for modulating ras gene expression in cells and tissues using the oligonucleotides or oligonucleotide analogs provided, and for specific modulation of expression of the activated ras gene. Methods for diagnosis, detection and treatment of conditions arising from the activation of the H-ras gene are also disclosed.		

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TC	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark	MG	Madagascar		
ES	Spain				

ANTISENSE OLIGONUCLEOTIDE INHIBITION OF THE ras GENE**FIELD OF THE INVENTION**

This invention relates to compositions and methods for the inhibition of expression of the ras gene, a naturally occurring gene which occasionally converts to an activated form which has been implicated in tumor formation. This invention is also directed to the specific inhibition of expression of the activated form of the ras gene. This invention is further directed to the detection of both normal and activated forms of the ras gene in cells and tissues, and can form the basis for research reagents and kits both for research and diagnosis. Furthermore, this invention is directed to treatment of such conditions as arise from activation of the ras gene.

BACKGROUND OF THE INVENTION

Alterations in the cellular genes which directly or indirectly control cell growth and differentiation are considered to be the main cause of cancer. There are some thirty families of genes, called oncogenes, which are implicated in human tumor formation. Members of one such family, the ras gene family, are frequently found to be mutated in human tumors. In their normal state, proteins produced by the ras genes are thought to be involved in normal cell growth and maturation. Mutation of the ras gene, causing an amino acid alteration at one of three critical positions in the protein product, results in conversion to a form which is implicated in tumor formation. A gene having such a mutation is said to be "activated." It is thought that such a point mutation leading to ras activation can be induced by

- 2 -

carcinogens or other environmental factors. Over 90% of pancreatic adenocarcinomas, about 50% of adenomas and adenocarcinomas of the colon, about 50% of adenocarcinomas of the lung and carcinomas of the thyroid, and a large fraction of malignancies of the blood such as acute myeloid leukemia and myelodysplastic syndrome have been found to contain activated ras oncogenes. Overall, some 10 to 20% of human tumors have a mutation in one of the three ras genes (H-ras, K-ras, or N-ras).

It is presently believed that inhibiting expression of activated oncogenes in a particular tumor cell might force the cell back into a more normal growth habit. For example, Feramisco et al., *Nature*, 314:639-642, 1985, demonstrated that if cells transformed to a malignant state with an activated ras gene are microinjected with antibody which binds to the protein product of the ras gene, the cells slow their rate of proliferation and adopt a more normal appearance. This has been interpreted as support for the involvement of the product of the activated ras gene in the uncontrolled growth typical of cancer cells.

Antisense oligonucleotide inhibition of oncogenes has proven to be a useful tool in understanding the roles of various oncogene families. "Antisense oligonucleotides" refers to small oligonucleotides which are complementary to the "sense" or coding strand of a given gene, and as a result are also complementary to, and thus able to specifically hybridize with, the mRNA transcript of the gene. Holt et al., *Mol. Cell Biol.*, 8, 963-973, 1988, have shown that antisense oligonucleotides hybridizing specifically with mRNA transcripts of the oncogene c-myc, when added to cultured HL60 leukemic cells, inhibit proliferation and induce differentiation. Anfossi et al., *Proc. Natl. Acad. Sci.*, 86, 3379-3383, 1989, have shown that antisense oligonucleotides specifically hybridizing with mRNA transcripts of the c-myc oncogene inhibit proliferation of human myeloid leukemia cell lines. Wickstrom et al., *Proc. Nat. Acad. Sci.*, 85, 1028-1032, 1988, have shown that expression of the protein product of the

- 3 -

c-myc oncogene as well as proliferation of HL60 cultured leukemic cells are inhibited by antisense oligonucleotides hybridizing specifically with c-myc mRNA. U.S. Patent 4,871,838 (Bos et al.) discloses oligonucleotides complementary to a mutation in codon 13 of N-ras to detect said mutation.

In all these cases, instability of unmodified oligonucleotides has been a major problem, as they are subject to degradation by cellular enzymes. PCT/US88/01024 (Zon et al.) discloses phosphorothioate oligonucleotide analogs hybridizable to the translation initiation region of the amplified c-myc oncogene to inhibit HL-60 leukemia cell growth and DNA synthesis in these cells. Tidd et al., *Anti-Cancer Drug Design*, 3, 117-127, 1988, evaluated antisense oligonucleotide methylphosphonate analogs hybridizing specifically to the activated N-ras oncogene and found that while they were resistant to biochemical degradation and were nontoxic in cultured human HT29 cells, they did not inhibit N-ras gene expression and had no effect on these cells. Chang et al., *Anti-Cancer Drug Design*, 4, 221-232, 1989, showed that both methylphosphonate and phosphorothioate analogs of oligonucleotides hybridizing specifically to mRNA transcripts of the Balb-ras gene could inhibit translation of the protein product of this gene *in vitro*. Because the antisense oligonucleotides and oligonucleotide analogs used by Chang et al. hybridize specifically with the translation initiation region of the ras gene, the binding ability of these oligonucleotides to normal (wild-type) vs. mutated (activated) ras genes was not compared.

The H-ras gene has recently been implicated in a serious cardiac arrhythmia called long Q-T syndrome, a hereditary condition which often causes sudden death if treatment is not given immediately. Frequently there are no symptoms prior to the onset of the erratic heartbeat. Whether the H-ras gene is precisely responsible for long Q-T syndrome is unclear. However, there is an extremely high correlation between inheritance of this syndrome and the presence of a

- 4 -

particular variant of the chromosome 11 region surrounding the H-ras gene. This makes the H-ras gene an excellent indicator of increased risk of sudden cardiac death due to the long Q-T syndrome.

5 There is a great desire to provide compositions of matter which can modulate the expression of the ras gene, and particularly to provide compositions of matter which specifically modulate the expression of the activated form of the ras gene. It is greatly desired to provide methods of
10 diagnosis and detection of the ras gene in animals. It is also desired to provide methods of diagnosis and treatment of conditions arising from ras gene activation. In addition, improved research kits and reagents for detection and study of the ras gene are desired.

15 **OBJECTS OF THE INVENTION**

 It is an object of the invention to provide oligonucleotides and oligonucleotide analogs which are capable of specifically hybridizing with RNA or DNA deriving from the mammalian ras gene.

20 It is a further object to provide oligonucleotides and oligonucleotide analogs which are capable of modulating the expression of the ras gene through antisense interaction with the mRNA product of the gene.

 Another object of the invention is to provide
25 oligonucleotides and oligonucleotide analogs which are capable of hybridizing selectively to the mRNA of the activated mutant form of the ras gene.

 Specific inhibition of expression of the activated form of the ras gene through hybridization of oligonucleotide
30 or oligonucleotide analogs with the mutated codon-12 region of the ras mRNA is yet another object of the invention.

 Detection of the mutation from the normal (wild-type) to activated form of the ras gene is another object of the invention.

35 Differential diagnosis of morphologically similar tumors and identification of high-risk conditions based on the presence of the activated ras gene is yet another object of

- 5 -

this invention.

A further object of this invention is to provide methods of diagnosis and treatment of conditions arising due to mutation of the gene from the wild-type to the mutant, activated form of the ras gene.

SUMMARY OF THE INVENTION

In accordance with the present invention, oligonucleotides and oligonucleotide analogs are provided that are specifically hybridizable with DNA or RNA deriving from the human ras gene. The oligonucleotide comprises nucleotide units sufficient in identity and number to effect such specific hybridization. It is preferred that the oligonucleotides or oligonucleotide analogs be specifically hybridizable with the translation initiation codon of the gene, and preferably that the oligonucleotide comprise a sequence CAT. In accordance with another preferred embodiment, oligonucleotides and oligonucleotides that specifically hybridize with codon 12 of the activated H-ras gene are provided, preferably comprising a sequence GAC. In another such embodiment, oligonucleotide or oligonucleotide analogs are provided that specifically hybridize preferentially with the mutated codon 12 of the activated H-ras gene. In this embodiment, such oligonucleotide or oligonucleotide analog preferably comprises a sequence GAC. Such oligonucleotides are conveniently and desirably presented in a pharmaceutically acceptable carrier.

In accordance with other preferred embodiments, the oligonucleotides and oligonucleotide analogs are formulated such that at least some of the linking groups between nucleotide units of the oligonucleotide comprise sulfur-containing species such as phosphorothioate moieties.

Other aspects of the invention are directed to methods for modulating the expression of the human ras gene in cells or tissues and for specifically modulating the expression of the activated ras gene in cells or tissues suspected of harboring a mutation leading to such activation. Additional aspects of the invention are directed to methods

- 6 -

of detection of the ras gene in cells or tissues and specific detection of the activated ras gene in cells or tissues suspected of harboring said mutated gene. Such methods comprise contacting cells or tissues suspected of containing
5 the human ras gene with oligonucleotides or oligonucleotide analogs in accordance with the invention in order to interfere with the effect of or detect said gene.

Other aspects of the invention are directed to methods for diagnostics and therapeutics of animals suspected
10 of having a mutation leading to activation of the ras gene. Such methods comprise contacting the animal or cells or tissues or a bodily fluid from the animal with oligonucleotides or oligonucleotide analogs in accordance with the invention in order to modulate the expression of this
15 gene, to treat conditions arising from activation of this gene, or to effect a diagnosis thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a bar graph showing dose-response inhibition of ras-luciferase fusion protein expression using
20 oligonucleotides targeted to the H-ras translation initiation codon (AUG). Expression is measured by measurement of luciferase activity as assayed by amount of light emitted when luciferin is added.

Figure 2 is a bar graph showing dose-response
25 inhibition of ras-luciferase fusion protein expression using oligonucleotides targeted to the mutated codon-12 region in activated H-ras. Expression is measured by measurement of luciferase activity as assayed by amount of light emitted when luciferin is added.

Figure 3 is a bar graph showing single-dose
30 inhibition of ras-luciferase fusion protein expression by antisense phosphorothioate compounds. Expression is measured by measurement of luciferase activity as assayed by amount of light emitted when luciferin is added.

Figure 4 is a table and bar graph summarizing data
35 obtained for 13 antisense oligonucleotides specifically hybridizable with the activated H-ras gene. Shown for each

- 7 -

oligonucleotide is its length, region of the activated ras gene to which it specifically hybridizes, and its activity in inhibiting expression of the ras-luciferase fusion protein.

DETAILED DESCRIPTION OF THE INVENTION

5 Malignant tumors develop through a series of stepwise, progressive changes that lead to the loss of growth control characteristic of cancer cells, i.e., continuous unregulated proliferation, the ability to invade surrounding tissues, and the ability to metastasize to different organ
10 sites. Carefully controlled *in vitro* studies have helped define the factors that characterize the growth of normal and neoplastic cells and have led to the identification of specific proteins that control cell growth and differentiation. In addition, the ability to study cell
15 transformation in carefully controlled, quantitative *in vitro* assays has led to the identification of specific genes capable of inducing the transformed cell phenotype. Such cancer-causing genes, or oncogenes, are believed to acquire transformation-inducing properties through mutations leading
20 to changes in the regulation of expression of their protein products. In some cases such changes occur in non-coding DNA regulatory domains, such as promoters and enhancers, leading to alterations in the transcriptional activity of oncogenes, resulting in over- or under-expression of their gene products.
25 In other cases, gene mutations occur within the coding regions of oncogenes, leading to the production of altered gene products that are inactive, overactive, or exhibit an activity that is different from the normal (wild-type) gene product.

To date, more than 30 cellular oncogene families
30 have been identified. These genes can be categorized on the basis of both their subcellular location and the putative mechanism of action of their protein products. The ras oncogenes are members of a gene family which encode related proteins that are localized to the inner face of the plasma
35 membrane. ras proteins have been shown to be highly conserved at the amino acid level, to bind GTP with high affinity and specificity, and to possess GTPase activity. Although the

- 8 -

cellular function of ras gene products is unknown, their biochemical properties, along with their significant sequence homology with a class of signal-transducing proteins known as GTP binding proteins, or G proteins, suggest that ras gene products play a fundamental role in basic cellular regulatory functions relating to the transduction of extracellular signals across plasma membranes.

Three ras genes, designated H-ras, K-ras, and N-ras, have been identified in the mammalian genome. Mammalian ras genes acquire transformation-inducing properties by single point mutations within their coding sequences. Mutations in naturally occurring ras oncogenes have been localized to codons 12, 13, and 61. The most commonly detected activating ras mutation found in human tumors is in codon 12 of the H-ras gene in which a base change from GGC to GTC results in a glycine-to-valine substitution in the GTPase regulatory domain of the ras protein product. This single amino acid change is thought to abolish normal control of ras protein function, thereby converting a normally regulated cell protein to one that is continuously active. It is believed that such deregulation of normal ras protein function is responsible for the transformation from normal to malignant growth.

The present invention provides oligonucleotides and oligonucleotide analogs for inhibition of human ras gene expression. The invention also provides oligonucleotides and oligonucleotide analogs for selective inhibition of expression of the mutant form of ras.

In the context of this invention, the term "oligonucleotide" refers to a plurality of joined nucleotide units formed from naturally-occurring bases and furanosyl groups joined by native phosphodiester bonds. This term effectively refers to naturally-occurring species or synthetic species formed from naturally-occurring subunits.

"Oligonucleotide analog," as that term is used in connection with this invention, refers to moieties which function similarly to oligonucleotides but which have non-naturally occurring portions. Thus, oligonucleotide analogs

- 9 -

may have altered sugar moieties or inter-sugar linkages. Exemplary among these are the phosphorothioate and other sulfur-containing species which are known for use in the art. They may also comprise altered base units or other
5 modifications consistent with the spirit of this invention.

In accordance with certain preferred embodiments, at least some of the phosphodiester bonds of the oligonucleotide have been substituted with a structure which functions to enhance the ability of the compositions to
10 penetrate into the region of cells where the RNA whose activity is to be modulated is located, and to make the compositions more resistant to degradation by cellular enzymes. It is preferred that such linkages be sulfur-containing. It is presently preferred that such substitutions
15 comprise phosphorothioate bonds. Others such as alkyl phosphothioate bonds, N-alkyl phosphoramidates, phosphorodithioates, alkyl phosphonates, and short chain alkyl or cycloalkyl structures may also be useful. In accordance with other preferred embodiments, the phosphodiester bonds are
20 substituted with structures which are, at once, substantially non-ionic and non-chiral, or, alternatively, with structures that are sequence-specific and substantially chiral. Persons of ordinary skill in the art will be able to select other linkages for use in the practice of the invention.

25 Oligonucleotide analogs may also include species which include at least some modified base forms. Thus, purines and pyrimidines other than those normally found in nature may be so employed. In accordance with one such embodiment, one or more bases comprises 2-(amino)adenine. In
30 other such embodiments, one or more bases comprises 2-(methylamino)adenine, 2-(alkylamino)adenine, 2-(imidazolylalkyl)adenine, 2-(aminoalkylamino)adenine or other heterosubstituted alkyladenines. Similarly, modifications on the furanose portions of the nucleotide subunits may also
35 occur as long as the essential tenets of this invention are adhered to.

Such analogs are best described as being

- 10 -

functionally interchangeable with natural oligonucleotides (or synthesized oligonucleotides along natural lines), but which have one or more differences from natural structure. All such analogs are comprehended by this invention so long as they function effectively to hybridize with the ras gene or mRNA deriving from it to inhibit the function or expression of the ras gene.

The oligonucleotides and oligonucleotide analogs in accordance with this invention preferably comprise from about 10 to about 30 subunits. It is more preferred that such oligonucleotides and analogs comprise from about 15 to about 25 subunits. As will be appreciated, a subunit is a base and sugar combination suitably bound to adjacent subunits through phosphodiester or other bonds.

The oligonucleotides and oligonucleotide analogs of this invention are designed to be hybridizable with messenger RNA derived from the H-ras gene. Such hybridization, when accomplished, interferes with the normal roles of the messenger RNA to cause a loss of its function in the cell. The functions of messenger RNA to be interfered with include all vital functions such as translocation of the RNA to the site for protein translation, actual translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and possibly even independent catalytic activity which may be engaged in by the RNA. The overall effect of such interference with the RNA function is to interfere with expression of the H-ras gene. The protein products of the other mammalian ras genes, N-ras and K-ras, are identical to H-ras over the first 85 amino acids. The nucleic acid sequences of the three ras genes, while not identical, are known, and persons of ordinary skill in the art will be able to use this invention as a guide in preparing oligonucleotides or oligonucleotide analogs specifically hybridizable with the N-ras and K-ras genes.

The oligonucleotides and oligonucleotide analogs of this invention can be used in diagnostics, therapeutics and as research reagents and kits. Since the oligonucleotides and

- 11 -

oligonucleotide analogs of this invention hybridize to the ras gene, sandwich and other assays can easily be constructed to exploit this fact. Furthermore, since the oligonucleotides and oligonucleotide analogs of this invention hybridize preferentially to the mutant (activated) form of the ras oncogene, such assays can be devised for screening of cells and tissues for ras conversion from wild-type to activated form. Such assays can be utilized for differential diagnosis of morphologically similar tumors, and for detection of increased risk of cancer stemming from ras gene activation. Provision of means for detecting hybridization of oligonucleotide or analog with the ras gene can routinely be accomplished. Such provision may include enzyme conjugation, radiolabelling or any other suitable detection systems. Kits for detecting the presence or absence of ras or activated ras may also be prepared.

The following examples illustrate the present invention and are not intended to limit the same.

EXAMPLES

20 Example 1

Oligonucleotide synthesis: Unmodified oligonucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidate chemistry with oxidation by iodine. For phosphorothioate oligonucleotides, the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 hr), the oligonucleotides were purified by precipitation twice out of 0.5 M NaCl solution with 2.5 volumes ethanol. Analytical gel electrophoresis was accomplished in 20% acrylamide, 8 M urea, 454 mM Tris-borate buffer, pH=7.0. Oligonucleotides and phosphorothioates were judged from polyacrylamide gel electrophoresis to be greater than 80% full-length material.

- 12 -

Example 2

ras-Luciferase Reporter Gene Assembly: The ras-luciferase reporter genes described in this study were assembled using PCR technology. Oligonucleotide primers were synthesized for use as primers for PCR cloning of the 5'-regions of exon 1 of both the mutant (codon 12) and non-mutant (wild-type) human H-ras genes. H-ras gene templates were purchased from the American Type Culture Collection (ATCC numbers 41000 and 41001) in Bethesda, MD. The oligonucleotide PCR primers 5'-ACA-TTA-TGC-TAG-CTT-TTT-GAG-TAA-ACT-TGT-GGG-GCA-GGA-GAC-CCT-GT-3' (sense), SEQ ID NO: 7, and 5'-GAG-ATC-TGA-AGC-TTC-TGG-ATG-GTC-AGC-GC-3' (antisense), SEQ ID NO: 8, were used in standard PCR reactions using mutant and non-mutant H-ras genes as templates. These primers are expected to produce a DNA product of 145 base pairs corresponding to sequences -53 to +65 (relative to the translational initiation site) of normal and mutant H-ras, flanked by NheI and HindIII restriction endonuclease sites. The PCR product was gel purified, precipitated, washed and resuspended in water using standard procedures.

PCR primers for the cloning of the *P. pyralis* (firefly) luciferase gene were designed such that the PCR product would code for the full-length luciferase protein with the exception of the amino-terminal methionine residue, which would be replaced with two amino acids, an amino-terminal lysine residue followed by a leucine residue. The oligonucleotide PCR primers used for the cloning of the luciferase gene were 5'-GAG-ATC-TGA-AGC-TTG-AAG-ACG-CCA-AAA-ACA-TAA-AG-3' (sense), SEQ ID NO: 9, and 5'-ACG-CAT-CTG-GCG-CGC-CGA-TAC-CGT-CGA-CCT-CGA-3' (antisense), SEQ ID NO: 10, were used in standard PCR reactions using a commercially available plasmid (pT3/T7-Luc) (Clontech), containing the luciferase reporter gene, as a template. These primers were expected to yield a product of approximately 1.9 kb corresponding to the luciferase gene, flanked by HindIII and BssHII restriction endonuclease sites. This fragment was gel purified, precipitated, washed and resuspended in water using

- 13 -

standard procedures.

To complete the assembly of the ras-luciferase fusion reporter gene, the ras and luciferase PCR products were digested with the appropriate restriction endonucleases and
5 cloned by three-part ligation into an expression vector containing the steroid-inducible mouse mammary tumor virus promoter MMTV using the restriction endonucleases NheI, HindIII and BssHII. The resulting clone results in the
10 insertion of H-ras 5' sequences (-53 to +65) fused in frame with the firefly luciferase gene. The resulting expression vector encodes a ras-luciferase fusion product which is expressed under control of the steroid-inducible MMTV promoter.

Example 3

15 Transfection of Cells with Plasmid DNA:

Transfections were performed as described by Greenberg, M.E., in Current Protocols in Molecular Biology, (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman and K. Strahl, eds.), John Wiley and Sons, NY, with the following
20 modifications. HeLa cells were plated on 60 mm dishes at 5×10^5 cells/dish. A total of 10 μ g of DNA was added to each dish, of which 9 μ g was ras-luciferase reporter plasmid and 1 μ g was a vector expressing the rat glucocorticoid receptor under control of the constitutive Rous sarcoma virus (RSV)
25 promoter. Calcium phosphate-DNA coprecipitates were removed after 16-20 hours by washing with Tris-buffered saline [50 mM Tris-Cl (pH 7.5), 150 mM NaCl] containing 3 mM EGTA. Fresh medium supplemented with 10% fetal bovine serum was then added to the cells. At this time, cells were pre-treated with
30 antisense oligonucleotides prior to activation of reporter gene expression by dexamethasone.

Example 4

Oligonucleotide Treatment of Cells: Immediately following plasmid transfection, cells were washed three times
35 with Opti-MEM (Gibco), prewarmed to 37°C. Two ml of Opti-MEM containing 10 μ g/ml N-[1-(2,3-dioleyloxy)propyl]-N,N,N,-trimethylammonium chloride (DOTMA) (Bethesda Research Labs,

- 14 -

Gaithersburg, MD) was added to each dish and oligonucleotides were added directly and incubated for 4 hours at 37°C. Opti-MEM was then removed and replaced with the appropriate cell growth medium containing oligonucleotide. At this time, reporter gene expression was activated by treatment of cells with dexamethasone to a final concentration of 0.2 μ M. Cells were harvested 12-16 hours following steroid treatment.

Example 5

Luciferase Assays: Luciferase was extracted from cells by lysis with the detergent Triton X-100, as described by Greenberg, M.E., in Current Protocols in Molecular Biology, (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman and K. Strahl, eds.), John Wiley and Sons, NY. A Dynatech ML1000 luminometer was used to measure peak luminescence upon addition of luciferin (Sigma) to 625 μ M. For each extract, luciferase assays were performed multiple times, using differing amounts of extract to ensure that the data were gathered in the linear range of the assay.

Example 6

Antisense Oligonucleotide Inhibition of ras-Luciferase Gene Expression: A series of antisense phosphorothioate oligonucleotide analogs targeted to either the H-ras translation initiation codon or the codon-12 point mutation of activated H-ras were screened using the ras-luciferase reporter gene system described in the foregoing examples. Of this initial series, six oligonucleotides were identified that gave significant and reproducible inhibition of ras-luciferase activity. The base sequences, sequence reference numbers and sequence ID numbers of these oligonucleotides (all are phosphorothioate analogs) are shown in Table 1.

- 15 -

TABLE 1

<u>Oligo ref. no.</u>	<u>Sequence</u>	<u>SEQ ID NO:</u>
2502	CTT-ATA-TTC-CGT-CAT-CGC-TC	1
2503	TCC-GTC-ATC-GCT-CCT-CAG-GG	2
5 2570	CCA-CAC-CGA-CGG-CGC-CC	3
2571	CCC-ACA-CCG-ACG-GCG-CCC-A	4
2566	GCC-CAC-ACC-GAC-GGC-GCC-CAC	5
2560	TGC-CCA-CAC-CGA-CGG-CGC-CCA-CC	6

Figure 1 shows a dose-response experiment in which
10 cells expressing either the normal ras-luciferase reporter
gene or the mutant ras-luciferase reporter gene were treated
with increasing concentrations of the phosphorothioate
oligonucleotide analog 2503 (sequence I.D. no. 2). This
compound is targeted to the translational initiation codon of
15 H-ras RNA transcripts. As shown in Figure 1, treatment of
cells with this oligonucleotide resulted in a dose-dependent
inhibition of ras-luciferase activity, displaying IC₅₀ values
of approximately 50 nM for both the normal and the mutant ras
targets. The control oligonucleotide is a random
20 phosphorothioate oligonucleotide analog, 20 bases long.
Results are expressed as percentage of luciferase activity in
transfected cells not treated with oligonucleotide. The
observation that an oligonucleotide targeted to the ras
translation initiation codon is equally effective in reducing
25 both mutant and normal ras expression is expected since the
two targets have identical sequence compositions in the region
surrounding the AUG translation initiation site.

Figure 2 shows a dose-response experiment in which
cells were treated with phosphorothioate oligonucleotide
30 analog 2570 (sequence I.D. no. 3), a compound that is targeted
to the codon-12 point mutation of mutant (activated) H-ras
RNA. The control oligonucleotide is a random phosphorothioate
oligonucleotide analog, 20 bases long. Results are expressed
as percentage of luciferase activity in transfected cells not
35 treated with oligonucleotide. As the figure shows, treatment
of cells with increasing concentrations of this

- 16 -

oligonucleotide resulted in a dose-dependent inhibition of ras-luciferase activity in cells expressing either the mutant form or the normal form of ras-luciferase. However, careful examination of the data shows that at low concentrations, oligonucleotide 2570 displayed approximately threefold selectivity toward the mutant form of ras-luciferase as compared to the normal form. In fact, 2570 displayed an IC₅₀ value for the mutant form of ras-luciferase of approximately 100 nM whereas the same compound displayed an IC₅₀ value of approximately 250 nM for the unmutated form.

Figure 3 shows the results of a typical experiment in which cells expressing either the normal form or the mutant form of ras-luciferase were treated with a single dose (0.5 μ M) of oligonucleotide targeted to either the translation initiation codon of H-ras or the codon-12 point mutation. The antisense phosphorothioate oligonucleotide analogs tested are shown in Table 1. The control oligonucleotide (2504) is a random phosphorothioate oligonucleotide analog, 20 bases long. Results are expressed as percentage of luciferase activity in transfected cells not treated with oligonucleotide. As shown in Figure 3, compound 2503 (sequence I.D. no. 2), targeted to the ras translational initiation codon, was most effective in inhibiting ras-luciferase activity. Of the three compounds targeted to the codon-12 point mutation of activated H-ras, only the 17-mer oligonucleotide 2570 (sequence I.D. no. 3) displayed selectivity toward the mutated form of ras-luciferase as compared to the normal form. This is also shown in Figure 4, which summarizes data obtained with all 13 antisense oligonucleotides complementary to the activated H-ras gene, as well as a scrambled control oligonucleotide (1966) and a control oligonucleotide (2907) complementary to the codon-12 region of wild-type ras. Shown for each oligonucleotide is its length, region to which it is complementary, and its activity in suppressing expression of the ras-luciferase fusion protein. The longer phosphorothioates targeted to the codon-12 point mutation, while displaying substantial antisense activity toward ras-

- 17 -

luciferase expression, did not demonstrate selective inhibition of expression of the mutant form of ras-luciferase. Phosphorothioate oligonucleotides targeted to the codon-12 point mutation that were less than 17 nucleotides in length did not show activity to either form of ras-luciferase. ~~These~~ results demonstrate effective antisense activity of phosphorothioate oligonucleotides targeted to ras sequences.

EXAMPLE 7

Synthesis of 2-(amino)adenine-substituted oligonucleotides:

Oligonucleotides and phosphorothioate oligonucleotide analogs will be synthesized as in Example 1, with the following exception: at positions at which a 2-(amino)adenine is desired, the standard phosphoramidite is replaced with a commercially available 2-aminodeoxyadenosine phosphoramidite (ChemGenes).

EXAMPLE 8

2-(Amino)adenine-Modified Antisense Oligonucleotide Inhibition of ras-Luciferase Gene Expression:

A series of antisense phosphorothioate oligonucleotide analogs complementary to the codon-12 point mutation of activated ras will be synthesized as described in Example 7, having a 2-(amino)adenine at the position complementary to the uracil of the mutated codon 12. Because the amino group at the 2-position of the adenine is able to hydrogen-bond with the oxygen in the 2-position on the uracil, three hydrogen bonds instead of the usual two are formed. This serves to greatly stabilize the hybridization of the 2-(amino)adenine-modified antisense oligonucleotide to the activated ras gene while destabilizing, or having no net effect, on the stability of this oligo to the wild-type codon 12, because of the modified A-G mismatch at this position.

- 18 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Monia, Brett P.
Freier, Susan M.
Ecker, David J.
- (ii) TITLE OF INVENTION: ANTISENSE OLIGONUCLEOTIDE
INHIBITION OF THE H-ras GENE
- (iii) NUMBER OF SEQUENCES: 10
- (iv) CORRESPONDENCE ADDRESS:
- 10 (A) ADDRESSEE: Woodcock Washburn Kurtz
Mackiewicz & Norris
- (B) STREET: One Liberty Place - 46th Floor
- (C) CITY: Philadelphia
- (D) STATE: PA
- 15 (E) COUNTRY: USA
- (F) ZIP: 19103
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE
- (B) COMPUTER: IBM PS/2
- 20 (C) OPERATING SYSTEM: PC-DOS
- (D) SOFTWARE: WORDPERFECT 5.0
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER: n/a
- (B) FILING DATE: herewith
- 25 (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (viii) ATTORNEY/AGENT INFORMATION:
- 30 (A) NAME: Jane Massey Licata

- 19 -

(B) REGISTRATION NUMBER: 32,257

(C) REFERENCE/DOCKET NUMBER: ISIS-0084

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (215) 568-3100

5 (B) TELEFAX: (215) 568-3439

(2) INFORMATION FOR SEQ ID NO:: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single stranded

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:: 1:

CTTATATTCC GTCATCGCTC

20

(2) INFORMATION FOR SEQ ID NO:: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

20 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single stranded

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

25 (iv) ANTI-SENSE: YES

- 20 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:: 2:

TCCGTCATCG CTCCTCAGGG

20

(2) INFORMATION FOR SEQ ID NO:: 3:

(i) SEQUENCE CHARACTERISTICS:

5

(A) LENGTH: 17

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single stranded

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

10

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:: 3:

CCACACCGAC GCGCCCC

17

(2) INFORMATION FOR SEQ ID NO:: 4:

15

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single stranded

(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:: 4:

CCCACACCGA CGGCGCCCA

19

- 21 -

(2) INFORMATION FOR SEQ ID NO:: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: nucleic acid

5 (C) STRANDEDNESS: single stranded

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:: 5:

GCCCCACACCG ACGGCGCCCA C

21

(2) INFORMATION FOR SEQ ID NO:: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23

15 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single stranded

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

20 (iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:: 6:

TGCCCCACACC GACGGCGCCC ACC

23

- 22 -

(2) INFORMATION FOR SEQ ID NO:: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 47

(B) TYPE: nucleic acid

5 (C) STRANDEDNESS: single stranded

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:: 7:

ACATTATGCT AGCTTTTTGA GTAACTTGT GGGGCAGGAG ACCCTGT

47

(2) INFORMATION FOR SEQ ID NO:: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29

15 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single stranded

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

20 (iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:: 8:

GAGATCTGAA GCTTCTGGAT GGTCAGCGC

29

- 23 -

(2) INFORMATION FOR SEQ ID NO:: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35

(B) TYPE: nucleic acid

5 (C) STRANDEDNESS: single stranded

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:: 9:

GAGATCTGAA GCTTGAAGAC GCCAAAAACA TAAAG

35

(2) INFORMATION FOR SEQ ID NO:: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33

15 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single stranded

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

20 (iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:: 10:

ACGCATCTGG CGCGCCGATA CCGTCGACCT CGA

33

- 24 -

CLAIMS

What is claimed is:

1. An oligonucleotide or oligonucleotide analog comprising from 10 to 30 nucleotide units specifically hybridizable with selected DNA or RNA deriving from the human H-ras gene.
2. The oligonucleotide or oligonucleotide analog of claim 1 specifically hybridizable with a translation initiation site or codon 12 of the human H-ras gene.
3. The oligonucleotide or oligonucleotide analog of claim 1 in a pharmaceutically acceptable carrier.
4. The oligonucleotide or oligonucleotide analog of claim 1 wherein at least some of the linking groups between nucleotide units of the oligonucleotide comprise sulfur-containing species.
5. The oligonucleotide or oligonucleotide analog of claim 4 wherein said sulfur-containing species comprises a phosphorothioate.
6. An oligonucleotide or oligonucleotide analog specifically hybridizable with selected DNA or RNA deriving from the human H-ras gene and comprising one of the sequences:
5'.....3'
CTT ATA TTC CGT CAT CGC TC, SEQ ID NO: 1;
TCC GTC ATC GCT CCT CAG GG, SEQ ID NO: 2;
CCA CAC CGA CGG CGC CC, SEQ ID NO: 3;
CCC ACA CCG ACG GCG CCC A, SEQ ID NO: 4;
GCC CAC ACC GAC GGC GCC CAC, SEQ ID NO: 5; and
TGC CCA CAC CGA CGG CGC CCA CC, SEQ ID NO: 6.

- 25 -

7. The oligonucleotide or oligonucleotide analog of claim 6 in a pharmaceutically acceptable carrier.

8. The oligonucleotide or oligonucleotide analog of claim 6 wherein at least some of the linking groups between
5 nucleotide units of the oligonucleotide comprise sulfur-containing species.

9. The oligonucleotide or oligonucleotide analog of claim 8 wherein said sulfur-containing species comprises a phosphorothioate.

10 10. A method of modulating the expression of the human H-ras gene comprising contacting tissues or cells containing the gene with an oligonucleotide or oligonucleotide analog comprising from 10 to 30 nucleotide units specifically hybridizable with selected DNA or RNA deriving from the human
15 H-ras gene.

11. The method of claim 10 wherein said oligonucleotide or oligonucleotide analog is specifically hybridizable with a translation initiation site or codon 12 of the human H-ras gene.

20 12. The method of claim 10 wherein said oligonucleotide or oligonucleotide analog comprises one of the sequences:

5'.....3'

CTT ATA TTC CGT CAT CGC TC, SEQ ID NO: 1;

25 TCC GTC ATC GCT CCT CAG GG, SEQ ID NO: 2;

CCA CAC CGA CGG CGC CC, SEQ ID NO: 3;

CCC ACA CCG ACG GCG CCC A, SEQ ID NO: 4;

GCC CAC ACC GAC GGC GCC CAC, SEQ ID NO: 5; and

- 26 -

TGC CCA CAC CGA CGG CGC CCA CC, SEQ ID NO: 6.

13. The method of claim 10 wherein at least some of the linking groups between nucleotide units of the oligonucleotide comprise sulfur-containing species.

5 14. The method of claim 13 wherein said sulfur-containing species comprises a phosphorothioate.

15. A method of detecting the presence of the H-ras gene in cells or tissues comprising contacting the cells or tissues with an oligonucleotide or oligonucleotide analog
10 comprising from 10 to 30 nucleotide units specifically hybridizable with selected DNA or RNA deriving from the human H-ras gene.

16. The method of claim 15 wherein said oligonucleotide or oligonucleotide analog is specifically
15 hybridizable with a translation initiation site or codon 12 of the human H-ras gene.

17. The method of claim 15 wherein said oligonucleotide or oligonucleotide analog comprises one of the sequences:

20 5'.....3'
CTT ATA TTC CGT CAT CGC TC, SEQ ID NO: 1;
TCC GTC ATC GCT CCT CAG GG, SEQ ID NO: 2;
CCA CAC CGA CGG CGC CC, SEQ ID NO: 3;
CCC ACA CCG ACG GCG CCC A, SEQ ID NO: 4;
25 GCC CAC ACC GAC GGC GCC CAC, SEQ ID NO: 5; and
TGC CCA CAC CGA CGG CGC CCA CC, SEQ ID NO: 6.

- 27 -

18. The method of claim 15 wherein at least some of the linking groups between nucleotide units of the oligonucleotide comprise sulfur-containing species.

19. The method of claim 18 wherein said sulfur-containing species comprises a phosphorothioate.

20. A method of detecting activated H-ras based on the differential affinity of particular oligonucleotides for activated vs. wild-type H-ras comprising contacting cells or tissues suspected of containing it with the oligonucleotide:
10 5' CCA-CAC-CGA-CGG-CGC-CC 3', SEQ ID NO: 3
and contacting an identical sample of cells or tissues with one of the oligonucleotides:

5'.....3'

CTT ATA TTC CGT CAT CGC TC, SEQ ID NO: 1;

15 TCC GTC ATC GCT CCT CAG GG, SEQ ID NO: 2;

CCC ACA CCG ACG GCG CCC A, SEQ ID NO: 4;

GCC CAC ACC GAC GGC GCC CAC, SEQ ID NO: 5; and

TGC CCA CAC CGA CGG CGC CCA CC, SEQ ID NO: 6.

21. The method of claim 20 wherein at least some of
20 the linking groups between nucleotide units of at least one of the oligonucleotides comprise sulfur-containing species.

22. The method of claim 21 wherein said sulfur-containing species comprises a phosphorothioate.

23. A method of treating conditions arising from the
25 activation of the H-ras oncogene comprising contacting an animal with an oligonucleotide or oligonucleotide analog comprising from 10 to 30 nucleotide units specifically hybridizable with selected DNA or RNA deriving from the human

- 28 -

H-ras gene.

24. The method of claim 23 wherein said oligonucleotide or oligonucleotide analog comprises at least a portion of the sequence: 5' CCA-CAC-CGA-CGG-CGC-CC 3', SEQ
5 ID NO: 3.

25. The method of claim 23 wherein at least some of the linking groups between nucleotide units of the oligonucleotide comprise sulfur-containing species.

26. The method of claim 25 wherein said sulfur-
10 containing species comprises a phosphorothioate.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/05008

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C12N 15/11, 15/12; A61K 48/00

US CL : 435/6, 172.1; 514/44; 536/27

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 172.1; 514/44; 536/27

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

search terms: H-ras, human, Q-T, antisense, phosphorothioate

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US, A, 4,871,838 (Bos et al) 03 October 1989, col. 1, lines 45-52, col. 5, line 16 - col. 6, line 27.	1-3 4-22
Y,P	US, A, 5,087,617 (Smith et al) 11 February 1992, col. 1, lines 9-24, col. 4, lines 11-14.	4-26
Y	Proc. Natl. Acad. Sci. USA, Volume 85, issued February 1988, E. L. Wickstrom et al, "Human Promyelocytic Leukemia HL-60 Cell Proliferation and c-myc Protein Expression are Inhibited by an Antisense Pentadecadeoxynucleotide Targeted Against c-myc mRNA", pages 1028-1032, see the entire document.	10-14
Y	Molecular and Cellular Biology, Volume 8, No. 2, issued February 1988, J. T. Holt et al, "An Oligomer Complementary to c-myc mRNA Inhibits Proliferation of HL-60 Promyelocytic Cells and Induces Differentiation", pages 963-973, see the entire document.	10-14

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Z" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

15 September 1992

Date of mailing of the international search report

28 SEP 1992

Name and mailing address of the ISA/
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer

NANCY VOGEL

Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/05008

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proc. Natl. Acad. Sci. USA, Volume 86, issued May 1989, G. Anfossi et al, "An Oligomer Complementary to c-myc-encoded mRNA inhibits Proliferation of Human Myeloid Leukemia Cell Lines", pages 3379-3383, see the entire document	10-14
Y	Anti-Cancer Drug Design, Volume 4, issued 1989, E. H. Chang et al, "Comparative Inhibition of ras p21 Protein Synthesis with Phosphorus-modified Antisense Oligonucleotides", pages 221-232, see the entire document.	4-26
Y	Anti-Cancer Drug Design, Volume 3, issued 1988, D. M. Tidd et al, "Evaluation of N-ras Oncogene Anti-sense, Sense and Nonsense Sequence Methylphosphonate Oligonucleotide Analogs", pages 117-127, see the entire document.	4-26
A	Science, Volume 252, issued 03 May 1991, M. Keating et al, "Linkage of a Cardiac Arrhythmia, the Long QT Syndrome, and the Harvey ras-1 Gene", pages 704-706, see the entire document.	1-26
<u>X</u>	Mutation Research, Volume 195, issued 1988, J. Bos, "The ras gene family and human carcinogenesis", pages 255-271, see the entire document.	<u>1-3</u>
Y		4-22

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.